CD24a Expression Levels Discriminate Langerhans Cells from Dermal Dendritic Cells in Murine Skin and Lymph Nodes

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Langerhans cells (LCs) and dermal dendritic cells (dDCs) are the professional antigen-presenting cells of the skin. Recently, their immunogenic *versus* tolerogenic role has come under re-investigation. LCs are distinguished from dDCs by Langerin (CD207) staining or by detection of Birbeck granules. However, for *in vitro* experiments it is desirable to have a simple and robust flow cytometric demarcation of both cell types. We show here that CD24a is expressed on LCs but not on dDCs isolated directly from the skin. Moreover, in combination with major histocompatibility complex class II (MHCII), CD24a expression levels distinguish LCs from dDCs in skin-draining lymph nodes after antigen activation and migration. High expression of CD24a correlated strictly with CD207 expression. MHCII^{high} cells were unique for skin-draining lymph nodes and were shown to be the only cells carrying antigen after FITC painting of the skin. CD24a expression levels further differentiated LCs and dDCs in the MHCII^{high} population. As staining for CD24a does not require fixation of cells, CD24a-stained cells can be used for *in vitro* experiments to analyze and compare the functional roles and properties of dDCs and LCs.

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INTRODUCTION

Dendritic cells (DCs) ensure transport of antigens captured from peripheral tissues to the draining secondary lymphoid organs, and are unique in their capacity to present antigen and prime naive T lymphocytes. At least seven different mouse CD11c$^+$ DC subsets have been identified endowed with different functional potential depending on their phenotype and environment (Ardavin, 2003). Two of them can be found in the skin and skin-draining lymph nodes (sdLNs).

Langerhans cells (LCs) reside in an immunologically immature state in the suprabasal layer of the epidermis, where they detect invading pathogens or antigens (Romani *et al.*, 2003). They are characterized by their unique Birbeck granules and Langerin expression (Valladeau *et al.*, 2000). LCs migrate as precursors from the blood through the dermis to the epidermis. After antigen uptake they mature and leave the skin through the dermis for the lymph node (LN) to initiate an immune response or, alternatively, exert tolerogenic functions (Steinman *et al.*, 2003). The dermis harbors a second DC subset, the dermal DC (dDC) or interstitial DC. dDCs were shown to be important for skin immunity. They are critically involved in the induction of an immune response against haptens in the absence of epidermal LCs (Bennett *et al.*, 2005; Kissenpfennig *et al.*, 2005), and initiate protective T-cell responses to certain viral antigens (Allan *et al.*, 2003, 2006). LCs and dDCs express a mature phenotype, characterized by high levels of major histocompatibility complex class II (MHCII) and costimulatory molecules, when they constitutively migrate at a low rate from the skin to sdLNs (Anjuere *et al.*, 1999; Hemmi *et al.*, 2001). This homeostatic turnover increases under inflammatory conditions. dDCs appear in the LN as early as 24 hours after skin sensitization, whereas LCs require 3–4 days to migrate from the epidermis into lymphoid organs (Kissenpfennig *et al.*, 2005).

Although it is known that MHCII-positive cells in the epidermis are LCs, it is challenging to differentiate MHCII-positive cells within the dermis, which comprise immature LCs, mature LCs, and dDCs. This is of importance since the unambiguous identification of LCs and dDCs is hampered by their overlapping surface-marker expression profiles. So far, only the differential expression of CD207 (Langerin) enables the discrimination between LCs and resident dDCs. During maturation, epidermal LCs internalize their Langerin from the surface. Therefore, complex techniques of fixation and

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Abbreviations: DC, dendritic cell; dDC, dermal dendritic cell; LC, Langerhans cell; LN, lymph node; MHCII, major histocompatibility complex class II; sdLN, skin-draining lymph node

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intracellular staining are necessary for LC identification, which eliminates the possibility of further *in vitro* experiments. To analyze and compare the functional roles of dDCs and LCs directly *ex vivo*, a simple and clear-cut discrimination of both populations is desirable.

CD24 or ''heat-stable antigen'' is a pleiotropic, small, variably glycosylated, glycosylphosphatidylinositol-linked protein molecule. In the immune system, it was first reported as a developmental marker in thymocytes and as a costimulatory molecule for T cells (Liu *et al.*, 1992). CD24a has been shown to be expressed by murine DCs (Inaba *et al.*, 1992) and was found to act as a costimulatory molecule on LCs (Enk and Katz, 1994). Here we demonstrate that the level of CD24a expression clearly distinguishes epidermal LCs from dDCs in the skin and sdLNs, and thus can be utilized to separate LCs from dDCs for functional experiments.

RESULTS

CD24a expression coincides strictly with CD207 (Langerin) expression on skin DCs

While the epidermis contains almost exclusively LCs, the DCs in the dermis may be a mixture of migrating LCs and dDCs. The procedure of isolating DCs from skin requires enzymatic digestion with trypsin. Trypsin treatment led to the degradation of CD11c from the cell surface within minutes (data not shown), whereas MHCII expression was insensitive to trypsin treatment (data not shown). Thus, CD11c, normally the approved marker for analyzing DCs in the mouse, is not useful to recognize DCs isolated from the skin.

We noted differential expression of CD24a in dermal *versus* epidermal MHCII$^+$ cells. In contrast to CD11c, trypsin did not affect CD24a expression (data not shown). Therefore, we stained epidermal cell suspensions for MHCII, CD207, and CD24a. As shown in Figure 1a, all epidermal MHCII/CD207-positive cells (that is, LCs) also stained for CD24a. As expected, there is no MHCII$^+$CD207$^-$ population in the epidermis. Keratinocytes and T cells were also reported to express CD24a. Figure S1 shows that 80% of keratinocytes and 12% of CD3$^+$ T cells are positive for CD24a in untreated skin, although the respective expression levels are lower than those of LCs. Simultaneous staining for MHCII and CD24a thus allows a very distinct discrimination of LCs in the epidermis.

In contrast, the dermis consists of different MHCII$^+$ populations (Figure 1b), such as migrating LCs, resident dDCs, monocytes, and macrophages. Accordingly, we detected a small (0.5%) MHCII$^+$ CD207$^+$ population and a larger (5%) MHCII$^+$CD207$^-$ population in dermal cell preparations. As shown in Figure 1b, virtually all MHCII$^+$ CD207$^+$ cells expressed CD24a (filled graph), whereas CD207$^-$ cells were CD24a negative (black line). This latter cell population contained approximately 20% CD14$^+$ cells, that is, monocytes/macrophages (data not shown). We conclude that the cell-surface marker CD24a is specific for LCs and can be used to distinguish LCs from resident dDCs in the skin.

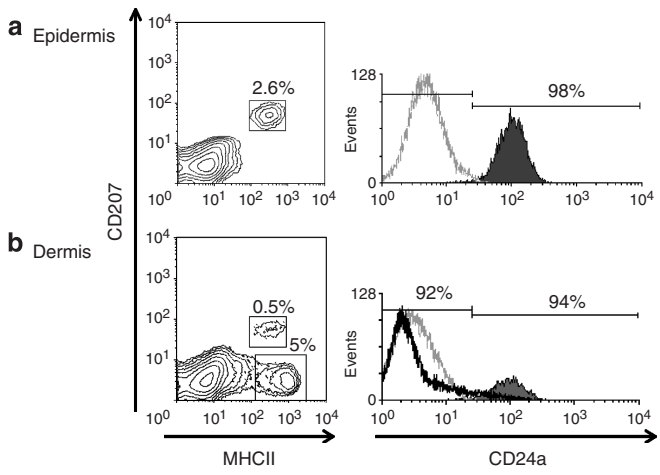

Figure 1. CD24a expression by DCs of the epidermis and dermis. Epidermis and dermis were separated by trypsin treatment and single-cell suspensions were analyzed by FACS. (**a**) Epidermal cells were stained for MHCII and CD207. Double positive cells were further analyzed for CD24a expression (filled graph). (**b**) CD24a expression in the dermis was analyzed in the population positive for MHCII and CD207 (filled graph), as well as in the MHCII$^+$ CD207$^-$ fraction (black line). Gray line represents the isotype control. Data are representative of three independent experiments.

CD24a expression levels distinguish dDCs and LCs in sdLNs

Distinguishing immigrated LCs *versus* dDCs in the draining LNs is important for studying skin-originated immune responses. Migration to the LNs is accompanied by complex processes of maturation of the DCs. We asked whether CD24a could be used to distinguish LCs from dDCs after migration to the LNs and stained sdLNs for MHCII, CD207, and CD24a. As shown in Figure 2a, sdLNs harbored a population of approximately 1% MHCII^{high} cells in contrast to mesenteric LN. To determine whether these cells represent skin-derived dendritic cell (sdDCs) (that is, LCs or dDCs), we tracked them by painting a solution of FITC on the ventral skin. Figure 2a shows that all FITC$^+$ cells could be found within the fraction of MHCII^{high} cells. Counterstaining with CD24a revealed two populations, namely a CD24a^{high} and a CD24a$^{low/int}$ population, within the FITC$^+$MHCII^{high} cells (Figure 2b). Over time the percentage of CD24a^{high} cells increased up to 75% of all MHCII^{high} cells. Similar to CD24a, the percentage of Langerin-positive cells increased to 75% during the observation period (Figure 2c and f). Double staining the cells for CD24a and Langerin showed that only the CD24a^{high} cells were CD207$^+$, whereas the CD24a$^{low/int}$ cells were CD207$^-$ (Figure 2d). Figure 2e shows the staining pattern of the whole LN cell suspension. The MHCII^{high} CD24a^{high} and MHCII^{high}CD24a$^{low/int}$ cells can be easily distinguished. Thus, dDCs appear to upregulate CD24a during maturation and migration from the skin to the LNs, albeit expression remains much lower than on LCs. Note also that CD207$^+$ LCs in the LNs express CD24a at a higher level than in the skin (compare Figures 1 and 2b); the mean fluorescence increases from 100 to 1,500 (Figure 2g).

Kinetics of CD24a^{high} cells immigrated from skin follows the pattern known for dDCs and LCs

We analyzed the expression of CD24a/CD207 on cells in the sdLNs up to 4 days after FITC sensitization of the skin.

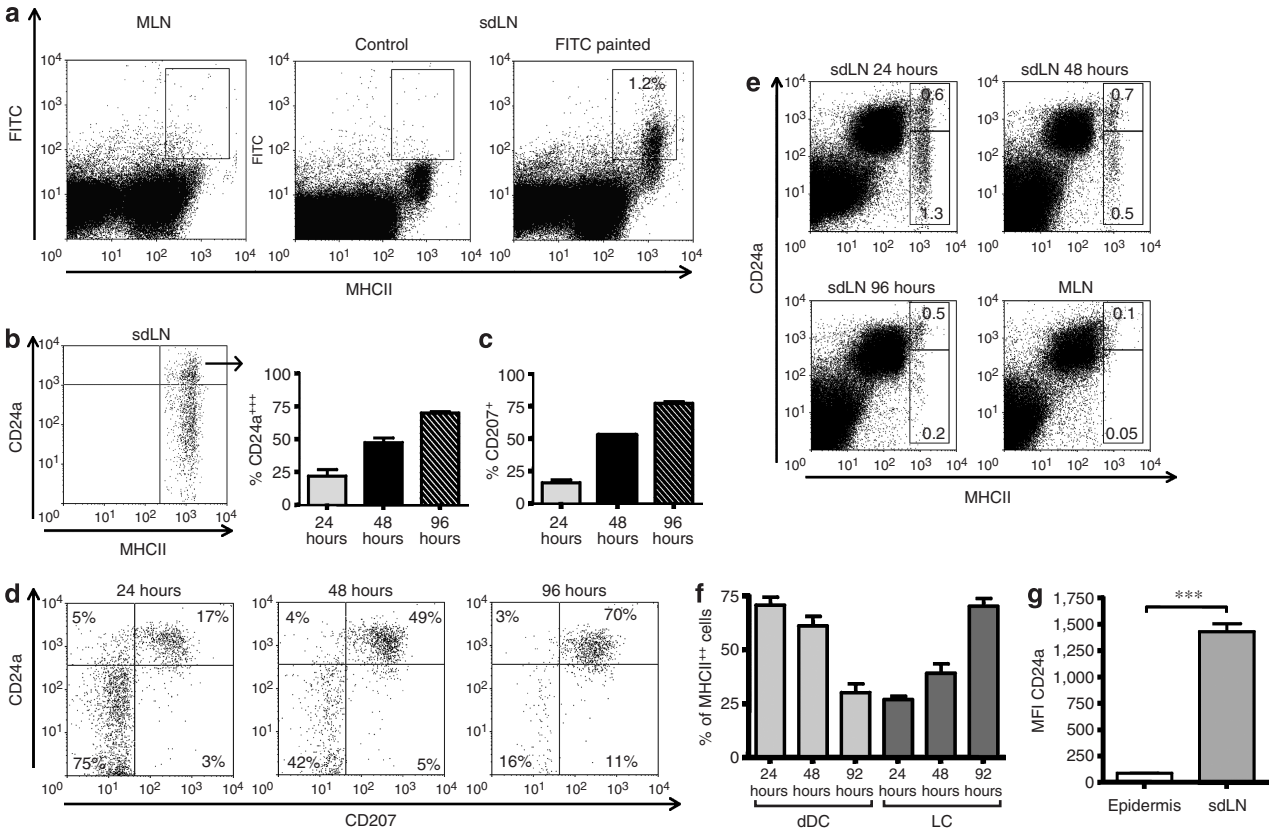

Figure 2. CD24a expression by sdDCs in LNs. Mice were shaved on the ventral skin and painted with FITC or solvent as control. A total of 24, 48, and 96 hours after application, sdLNs and mesenteric LNs were harvested and cells were analyzed by FACS. (**a**) sdDCs can be identified as FITC-positive MHCII^{high}. Shown are the dot plots of cells isolated 24 hours after application of FITC or solvent alone (control). (**b**) The FITC^{+} MHCII^{high} cells were analyzed for CD24a expression. The dot plot shows a representative CD24a staining pattern of MHCII^{high} cells 24 hours after FITC application. The percentage of CD24^{high} cells was calculated for all time points ($n=3$). (**c**) After staining of lymph node cells with Langerin and MHCII, the frequency of Langerin^{+} cells within the FITC^{+} MHCII^{high} fraction was calculated for all different time points after FITC application. (**d**) The dot plots are representative for the expression of CD24a and CD207 on FITC^{+} cells in sdLNs at the indicated time points. The frequencies shown in the figure are representative of three independent experiments. (**e**) The staining pattern of the whole sdLN cell suspension was analyzed at different time points. MHCII and CD24a expression is shown as well as the distribution of CD24a^{high} and CD24a$^{low/int}$ cells within the MHCII^{high} population. One out of three independent experiments is depicted here. (**f**) Frequency of CD24$^{low/int}$ dDCs and CD24a^{high} LCs within the MHCII^{high} cell population depending on the time after FITC application on the skin. (**g**) The mean fluorescence intensity of CD24a was compared between LCs in the epidermis and those in the sdLNs. *** indicates $P<0.0001$.

As shown in Figure 2b and d, the CD24a^{high} as well as the CD207^{+} population from all FITC^{+}MHCII^{high} cells in the LN increased from approximately 25% at day 1 to 75% at day 4, whereas the population of CD24a$^{low/int}$CD207^{-} cells decreased proportionately after day 1 (Figure 2f). This pattern confirms previous data on different migration kinetics of LCs and dDCs (Kissenpfennig *et al.*, 2005). Taken together, our data show that MHCII^{high}CD24^{high} staining discriminates LCs from dDCs in the LNs, while dDC can be identified by MHCII^{high}CD24$^{low/int}$ expression from other MHCII^{+} cells in the LNs.

CD24a expression on sdDCs *versus* blood-derived DCs in LNs

sdLNs harbor activated DCs, immigrated after antigenic activation, but also constitutively circulating DCs, which express CD11c, CD8α, and MHCII (Carbone *et al.*, 2004, and our own data, not shown). Also called ''blood derived'', some of these CD8α^{+} DCs can be CD207^{+} (Douillard *et al.*, 2005; Kissenpfennig *et al.*, 2005).

To determine whether CD207 expression on these cells also correlates with CD24a^{high} expression, we enriched DCs from sdLNs using a density gradient (Figure 3a). Of all CD11c^{+}CD8α^{+} cells, 12% were positive for CD207 (data not shown). Staining for CD24a and CD207 further showed that all blood-derived DCs, which are CD207^{+}, are indeed CD24a^{high} cells (gray-shaded histogram in Figure 3b). However, we also found some CD8α^{+}CD24a^{high} DCs that were negative for CD207 (thin line).

LCs and dDCs express high levels of CD40

The combination of CD11c and CD40 has been described previously to allow discrimination of LCs from dDCs after enrichment on a density gradient (Ruedl *et al.*, 2000). In order to confirm and extend this finding, we counterstained CD40/CD11c-stained LN cells with the specific marker CD207 after a density gradient enrichment. In this experiment we induced active immigration of skin DCs by sensitizing the abdominal

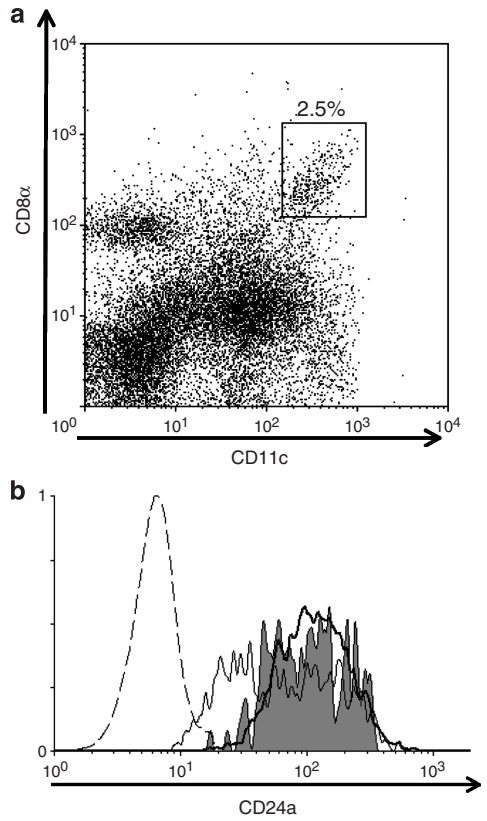

Figure 3. Blood-derived CD8α$^+$ DCs were analyzed for CD24a expression under steady-state conditions. sdLNs of untreated mice were harvested. DCs were enriched by density gradient centrifugation and stained for CD11c, CD8α, CD207, and CD24a. (**a**) Staining pattern for CD8α and CD11c after DC enrichment. The frequency of CD8α$^+$CD11c$^+$ DCs is indicated. (**b**) CD24a expression on gated CD11c$^+$CD8α$^+$CD207$^-$ cells (thin black line) and CD11c$^+$CD8α$^+$CD207$^+$ cells (gray filled histogram) is compared with that of all CD11c$^+$CD207$^+$ cells (thick black line) considered as control. The dotted line represents the isotype control.

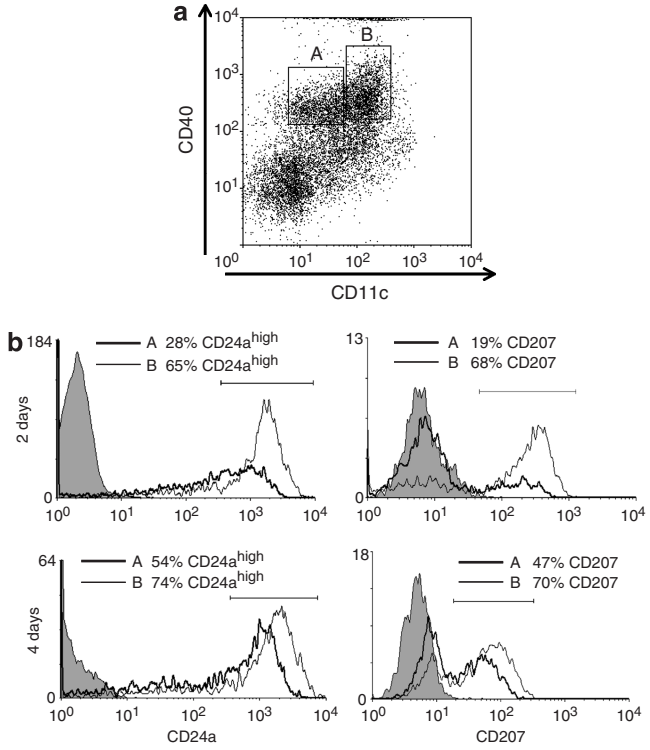

Figure 4. Comparison of the CD40/CD11c expression pattern with CD207/CD24a staining. Ventral skin of C57BL/6 mice was painted with 200 µl of 0.5% FITC to induce active migration of sdDCs to the sdLNs. After 48 and 96 hours, sdLNs were harvested and DCs were enriched by density-gradient centrifugation, and stained for CD40, CD11c, CD24a, and CD207. (**a**) The representative 48-hour dot plot depicts the CD11c$^+$CD40^{high} (A) and CD11c^{high}CD40^{high} (B) populations. These cells were further analyzed for CD207 and CD24a expression at the indicated time points. (**b**) The percentages of CD24a^{high} and CD207$^+$ cells were calculated for population A (thick black line) and B (thin black line).

skin with the hapten FITC, and analyzed draining LNs after 2 and 4 days for CD11c, CD40, CD207, and CD24a. Forty-eight and 96 hours after sensitization, we could identify a distinct difference in LC frequency between the populations A and B (Figure 4a) In both populations Langerin-expressing cells are present, the major being within the CD40^{high}CD11c^{high} cell fraction (almost 70%). However, since CD207$^+$CD24a^{high} cells were also present within population A, we conclude that staining for CD207 or CD24a is more suitable for discrimination of LCs and dDCs than the expression level of CD11c.

DISCUSSION

In the skin, at least two types of professional antigen-presenting cells are known, the LCs and the dDCs. Differential functions of these cell populations have been suggested, and lead to increased investigation into their tolerogenic *versus* immunogenic potential. Recently, inducible LC ablation was achieved by diphtheria toxin treatment of knock-in mice generated to express the diphtheria toxin receptor under the control of the CD207 promoter (Bennett *et al.*, 2005; Kissenpfennig *et al.*, 2005). This approach produced two different results. On the one hand, LCs were dispensable for triggering hapten-specific T-effector cells (Kissenpfennig *et al.*, 2005); on the other hand, contact hypersensitivity responses in LC-depleted mice were significantly decreased (Bennett *et al.*, 2005). In contrast, permanent ablation of LCs by direct transgenic diphtheria toxin expression in CD207$^+$ cells resulted in an enhanced contact hypersensitivity reaction (Kaplan *et al.*, 2005). These elegant experimental designs permit analysis of the function of dDCs in the absence of LCs and show that LCs might possess immunogenic as well as tolerogenic potential.

A technical difficulty in studying sdDCs is their isolation and phenotypic characterization *in situ*, *ex vivo*, and after migration to the LNs. Until now, the extra- and intracellular marker Langerin (CD207) has been used for LC identification. CD207 is a C-type lectin associated with the formation of Birbeck granules, which are unique for LCs. CD207 is a problematic marker as it is internalized during maturation and thus impossible to use for isolation of live cells. Although the identification of LCs is possible by staining for Langerin,

the differential detection of dDCs remains challenging (Valladeau and Saeland, 2005). Therefore, we looked for an alternative method to distinguish sdLCs from dDCs, which is difficult because of their overlapping phenotype (Anjuere *et al.*, 1999; Valladeau *et al.*, 2002). We here describe that the surface molecule CD24a is differentially expressed on LCs *versus* dDCs (i) within the skin and (ii) after migration to sdLNs. Since CD11c, the classical surface marker to identify DCs, is destroyed by trypsin treatment during isolation, we used MHCII expression to identify sdDCs. Sensitivity for trypsin was also described for DEC205 (Anjuere *et al.*, 1999), which is normally expressed more strongly by LCs than by dDCs.

It has been known for long that CD24a is expressed by various antigen-presenting cells, including DCs and LCs (Bruce *et al.*, 1981; Takei *et al.*, 1981; Inaba *et al.*, 1992), or by keratinocytes (Erdmann *et al.*, 1995; Figure S1). Already in 1994, Enk and Katz (1994) reported a costimulatory function of CD24a on LCs for T-helper 1 cell-dependent cutaneous immune reactions. However, they did not explore the use of CD24a in identification, tracking, or isolation of LCs *versus* dDCs from skin, or after migration to the LNs. Here, we report a strict correlation of CD24a with CD207 expression, and differential expression of CD24a in LCs *versus* dDCs. Other $CD24a^+$ cells such as activated T cells, which migrate into the skin, do not express MHCII. In any case, these and other $CD24a^+$ cells bear other lineage-specific surface molecules and are distinguishable from LCs and dDCs.

Some blood-derived DCs were described to be CD207 positive (Kissenpfennig *et al.*, 2005). We could demonstrate that this CD207 expression correlates with $CD24a^{high}$ expression. Although a fraction of $CD207^-$ blood-derived DCs is also $CD24a^{high}$, these cells are not $MHCII^{high}$ and can therefore easily be separated from sdDCs.

An alternative staining pattern for sdDCs was previously described by Ruedl *et al.* (2000), where $CD11c^{high}CD40^{high}$ cells were found to be LCs. After FITC painting of the skin, we could confirm that all FITC-positive cells were within the $CD40^{high}CD11c^+$ and $CD11c^{high}$ populations, but it was not possible to discriminate dDCs from LCs without additional staining. All FITC-positive cells were $MHCII^{high}$. This result is congruent with those of Ohl *et al.* (2004) who demonstrated that under steady-state conditions $MHCII^{high}$ cells are sdDCs. Additionally, the combination of CD24a and MHCII discriminated between LCs and dDCs.

Although different DC subsets display specific functional and phenotypic features, they share a characteristic phenotypic profile; in the mouse, conventional DCs are considered to be $MHCII^+$, $CD11c^+$, $CD40^+$, $CD86^+$, $CD3^-$, $B220^-$, Ig^-, and $Gr1^-$ (Anjuere *et al.*, 1999). We here add CD24a as a marker whose expression levels distinguish two DC populations in the skin and sdLNs. $CD207^+$ cells can also be found in spleen and mesenteric LNs, suggesting an uncharacterized population unrelated to epidermal LCs or a population of LCs with differing trafficking properties (Anjuere *et al.*, 1999; Valladeau *et al.*, 2002; Stoitzner *et al.*, 2003; Kissenpfennig *et al.*, 2005). Using CD24a as an additional marker in multi-parameter surface staining, it will be possible to analyze aberrant LC trafficking further.

Little is known about the regulation of CD24a expression. We noted increased expression of CD24a in LCs and dDCs after sensitization and migration to LNs. CD24a expression might be related to the migratory or homing behavior of LCs/dDCs, either as cause or effect. However, when LCs were taken in culture (where they mature), they exhibited reduced CD24a expression (Enk and Katz, 1994).

In conclusion, we have shown that the CD24a expression level in combination with MHCII is a distinguishing surface marker for skin-resident LCs and dDCs, as well as for activated LCs and dDCs in sdLNs. This method will be useful in analyzing the biology of LCs/dDCs *ex vivo* further. The functional role of differential CD24a expression, for example, in relation to the immunogenic and tolerogenic potential of these two cell types needs to be explored in future.

MATERIALS AND METHODS

Mice

Female C57BL/6 mice (Harlan Winkelmann GmbH, Borchen, Germany) were kept in the animal facility at the IUF, Düsseldorf, under specific pathogen-free conditions. Mice used were 7–12 weeks old.

Isolation of dDCs and epidermal LCs

The hair of the dorsal skin was removed with forceps and the skin sample was washed with 70% alcohol. Subcutaneous fat was scraped off with a scalpel. Skin pieces were placed on 0.25% trypsin/phosphate-buffered saline for 2 hours at 37°C. The epidermal layer was peeled from the underlying dermis and floated in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 5×10^{-5} M 2-mercaptoethanol, 0.15% (w/v) sodium hydrogen carbonate, 1 mM sodium pyruvate, 1 mM non-essential amino acids, 100 U ml^{-1} penicillin, and 100 µg ml^{-1} streptomycin (PAA Laboratories GmbH, Coelbe, Germany), hereafter referred to as complete medium. Epidermal cells were released by breaking up epidermal sheets with forceps, followed by vigorous pipetting. The dermis was further incubated for 40 minutes at 37°C with 0.5 mg ml^{-1} collagenase P (Roche, Mannheim, Germany) and 300 U ml^{-1} DNaseI (Roche) in RPMI-1640. The reaction was stopped with complete medium.

FITC painting

Mice were shaved on the abdomen and painted with 200 µl 0.5% FITC in acetone–dibutylphthalate (Sigma, Deisenhofen, Germany). After 24, 48, and 96 hours axillary, brachial, and inguinal LNs (referred to as sdLNs) were harvested. Mesenteric LNs were also harvested and served as negative control to detect the presence of LCs and dDCs. Single-cell suspensions were prepared from the LNs by incubation with collagenase D (Roche) and DNaseI (Roche) for 20 min. Cells were washed and stained for flow cytometry. Animal experiments were performed with permission from the Bezirksregierung Düsseldorf (Az.: 50.05-230-79/05).

Density gradient enrichment

LN cell suspensions were enriched for DCs by using the nycodenz gradient (Optiprep; Sigma) for 15 minutes at room temperature. The

method was performed as described before (Ruedl *et al.*, 2000). Cells were washed once and analyzed by flow cytometry.

Flow cytometry

The following monoclonal anti-mouse antibodies were used: anti-CD11c FITC (clone N418), anti-CD24a FITC and phycoerythrin (clone M1/69), anti-CD14 phycoerythrin (clone rmC5-3), anti-CD40 phycoerythrin (clone 3/23), anti-CD8α phycoerythrin (clone 53–6.7), and PerCP-conjugated streptavidin (from BD Biosciences, Heidelberg, Germany); anti-CD11c allophycocyanin (APC) (N418) and anti-I-A/I-E APC (clone M5/114.15.2) (from eBioscience), San Diego, CA); and biotinylated anti-CD207 (clone 926G4) (from AbCyS, Paris, France).

A total of 2×10^6 cells were preincubated with unconjugated anti-CD16/32 before staining to block Fc-receptors. For intracellular staining, cells were fixed with 2% paraformaldehyde (PFA) in phosphate-buffered saline and permeabilized by 0.5% saponin in 1% BSA in phosphate-buffered saline. All stainings were performed for 15 minutes at 4°C. Data were analyzed with a FACSCalibur in list mode, using CellQuest software and WinMDI. Live cells were gated using forward and sideward scatter.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure S1. CD24a expression of epidermal cells.

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